THROMBOSIS RESEARCH 15; 639-650
Pergamon Press Ltd.1979. Printed in Great Britain
0049-3848/79/0815-0639 $02.00/0

DECREASED PLATELET VITAMIN C IN DIABETES MELLITUS: POSSIBLE ROLE IN HYPERAGGREGATION

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(Received 8.11.1978; in revised form 27.3.1979.
Accepted by Editor K.M. Brinkhous)

ABSTRACT

To determine whether the handling of Vitamin C in the diabetic might be altered and might relate to the increased platelet sensitivity, we have investigated levels of platelet Vitamin C in the diabetic and determined the effects of Vitamin C in vitro or in vivo on platelet aggregation.

Levels of ascorbic acid, as tested by a lingual method, were significantly lower in diabetics than in normals ($p < .01$). Ascorbic acid levels in washed platelets from diabetics were significantly lower than from normals (45.2 ± 3 $\mu g/10^{10}$ platelets vs. 25.5 ± 2 $\mu g/10^{10}$ platelets, $p < .001$). The effects of ascorbic acid on platelet aggregation in vitro were studied by adding ascorbic acid in buffered solution (pH 7.35) prior to aggregating agents. Ascorbic acid (1000 µg/ml) in platelet-rich plasma consistently inhibited platelet aggregation with threshold concentrations of ADP, epinephrine, and collagen, but enhanced aggregation with arachidonic acid. With washed platelets, ascorbic acid inhibited arachidonic acid-induced aggregation. To rule out an interaction of ascorbic acid and arachidonic acid in the medium, platelets were incubated at 37°C for 10 minutes with varying concentrations of ascorbic acid, rewashed, and aggregation with arachidonic acid tested. Aggregation was inhibited in a linear dose-dependent fashion. Oral ingestion of ascorbic acid (2 gm/day) for seven days by normal non-smoking males produced a marked inhibition of aggregation. In a similar study, platelets from an insulin-dependent diabetic showed no change in aggregation. These results suggest that platelet levels of ascorbic acid may relate to the hyper-aggregation of platelets from diabetics.

INTRODUCTION

Diabetics are particularly prone to vascular disease with a greatly increased chance of developing heart attack, gangrene, kidney failure, and blindness (1). Platelets have been postulated to be involved in the development of both micro- and macrovascular disease (2).

Platelet adhesiveness and aggregation are clearly increased in diabetes. Platelet adhesiveness is increased with (3) or without added ADP (4-7). Colwell (8) and others (9-14) have reported an increased platelet aggregation in diabetes. Platelets from diabetics produce an increased amount of prostaglandin E-like material and at a faster rate than platelets from normal subjects (15).

Platelets have a very high content of ascorbic acid (20 times higher than plasma) (16) which is utilized during aggregation induced by thrombin, epinephrine, collagen, or ADP (17). Because of the molecular similarity of ascorbic acid to the sugars, we felt that its handling in the diabetic might be altered. We have, therefore, investigated levels of platelet Vitamin C in the diabetic and determined the effects of Vitamin C *in vitro* or *in vivo* on platelet aggregation.

MATERIALS AND METHODS

Lingual Vitamin C test

The lingual Vitamin C test was administered according to the method of Ringsdorf and Cheraskin (18). This involves the administration of a drop of the 2,6-dichloroindophenol sodium salt solution on the dorsum of the tongue at the junction of the anterior and middle 1/3 in a papillated area. The test utilizes an oxidation reduction reaction between the blue dye 2,6-dichloroindophenol and L ascorbic acid. The time it takes for the blue dye to become colorless is referred to as the "lingual time" and is a rough measure of the Vitamin C status of the individual. Lingual times of over two minutes were recorded as two minutes.

Platelet Vitamin C determinations

Blood (7 vol.) was drawn from fasting donors into 1% EDTA (1 vol.). Washed platelets were prepared by column chromatography according to the method of Tangen and Berman (19) except the eluting buffer was tris-saline. Platelet fractions were pooled, volume measured, and a platelet count taken (20). The washed platelets were spun down into a button, supernatant buffer discarded and .5 ml of 3% metaphosphoric acid with .0025 M EDTA added. This mixture was sonicated for 45 seconds and subsequently centrifuged at 1000 X g. The supernatant was decanted and Vitamin C measured using a modification of the method of Schmall (21). Diazotized 2-nitro-4-methoxy aniline is added to this supernatant. After allowing the tubes to stand 15 minutes, sodium hydroxide was added and tubes centrifuged for 20 minutes at 1000 X g to remove turbidity. Standards were prepared in the same manner using a known amount of Vitamin C ranging from 2.5 to 50 µg in a 3% metaphosphoric acid with .0025 M EDTA. The standard curve was highly reproducible from day to day and the color development linear over this range. Recoveries averaged 70%, so levels have been adjusted accordingly.

Platelet aggregation

For platelet aggregation, blood (9 vol.) was drawn into 3.8% sodium citrate (1 vol.) from fasting, normal donors and spun at 120 X g to obtain PRP. To 0.4 ml of PRP in a Payton aggregometer, 0.05 ml of Vitamin C in tris-saline dextrose (TSD, pH 7.35) or TSD was added followed by .05 ml of aggregating agent (ADP, epinephrine, collagen, or arachidonic acid).

For some experiments, arachidonic acid induced aggregation of column washed platelets was done. To the washed platelet suspension, a small amount of plasma was added (1/8 v:v).

Glucose determinations

Glucose was determined according to the method of Hoffman (22).

Donors

Subjects were male non-smokers who had not taken any drugs (other than insulin for diabetics) for at least ten days. Diabetics were all insulin-requiring. Informed consent was obtained.

Incubation experiments

Two experiments were done to determine whether the effect of Vitamin C was on the platelet or in the medium. For these experiments, column fractions containing washed platelets were pooled and divided into several 4.5 ml aliquots. Varying concentrations of Vitamin C in buffer (.5 ml) or buffer alone (.5 ml) were incubated with the aliquots at 37°C for 10 minutes. These platelets were then washed two times by centrifugation at 4°C for 10 minutes at 1000 X g, followed by resuspension in buffer. Final resuspension was in 1.3 ml tris-saline and .2 ml normal platelet-poor plasma. Arachidonic acid (.05 ml) was added to the final platelet suspension (.4 ml) in the aggregometer. The lowest concentration of arachidonic acid which produced full aggregation with the sample incubated with buffer was used to test aggregation in samples of platelets which had been incubated with Vitamin C. Heights of aggregometer curves four minutes after addition of arachidonic acid were measured.

In vivo experiments

Normal male non-smoking volunteers who had not taken any drugs for at least one week were used for these studies. Blood was drawn from fasting donors, and platelet aggregation in PRP and platelet Vitamin C levels were determined, before taking Vitamin C. The donor then took 2 gms of Vitamin C (500 mg q.i.d.) for 7 days. On day 8, a fasting sample was again tested for platelet aggregation and platelet Vitamin C levels.

RESULTS

Male diabetics (n = 20) from the Veterans Administration clinic had a mean lingual time of 27.9±4 (SEM) sec, which was significantly longer ($p < 0.02$) than the controls (17.2±1). This is compatible with the postulate of lower tissue Vitamin C levels in the diabetic.

is on the platelet rather than in the medium.

Oral ingestion of Vitamin C for one week by six normal non-smoking males

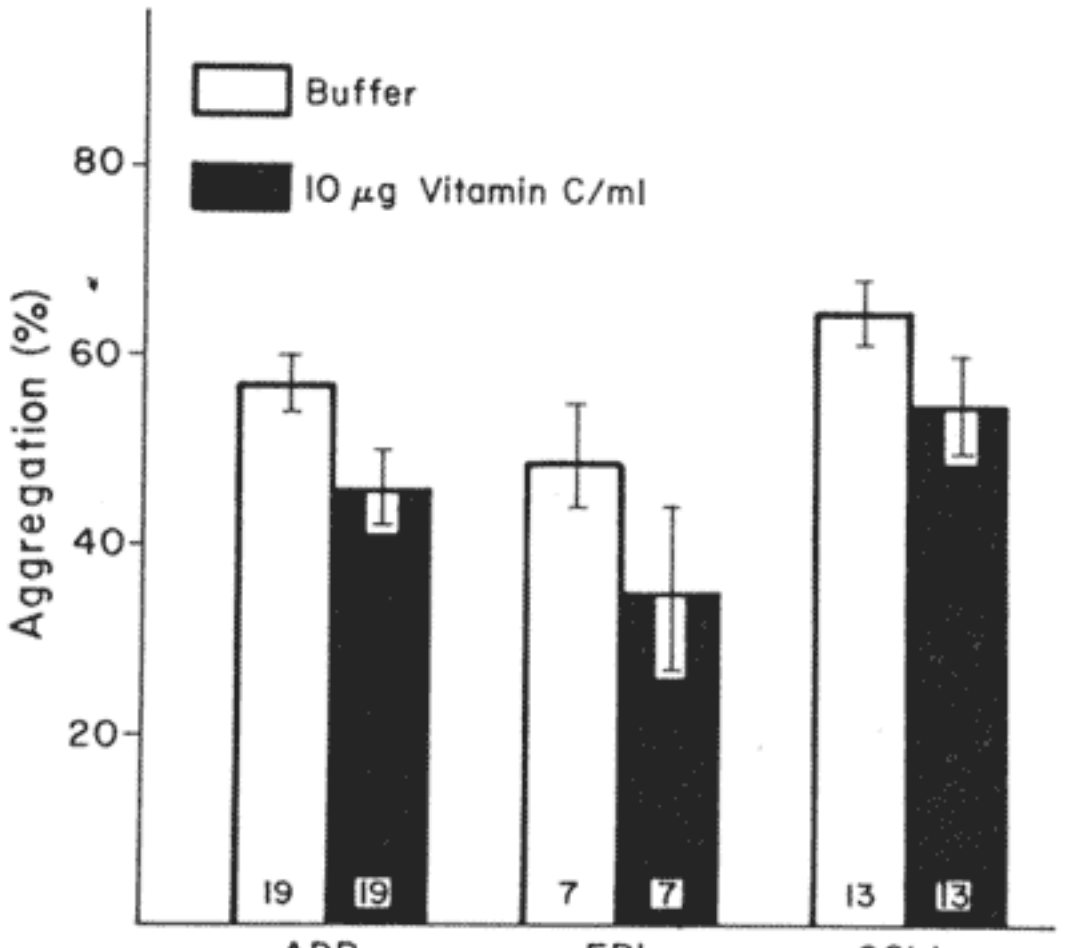

FIG. 2

The effect of 10 μg/ml Vitamin C *in vitro* on platelet aggregation.

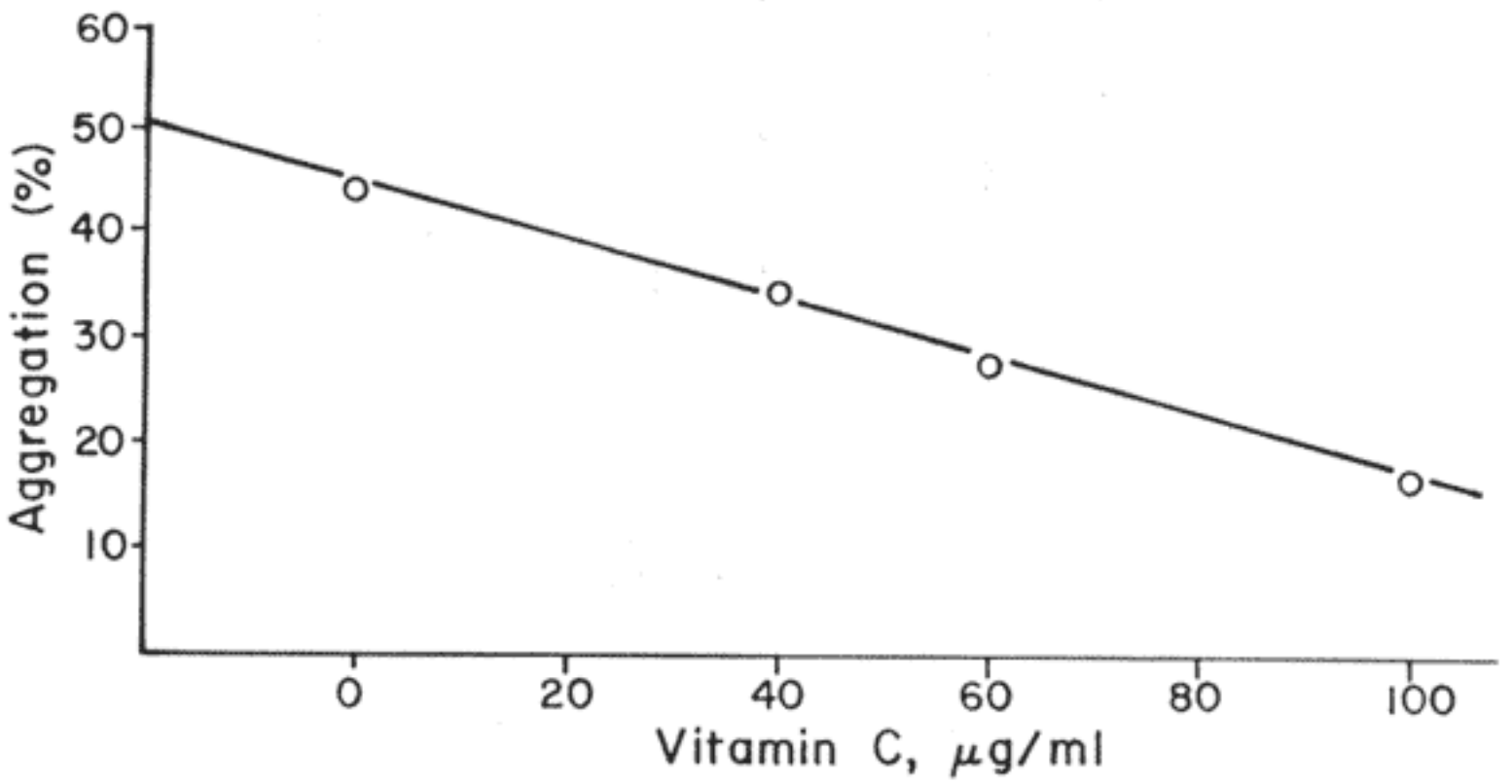

FIG. 3

Effect of incubation of platelets with Vitamin C on arachidonic acid-induced aggregation.

produced a significant inhibition of platelet aggregation (Figure 4). Heights of aggregometer curves before and after Vitamin C are shown in Table 1. Platelet levels of Vitamin C were measured in three of these donors. Levels rose from a mean of 47±17 $\mu g/10^{10}$ platelets before taking Vitamin C to 77±8 $\mu g/10^{10}$ platelets after Vitamin C. In one normal donor, platelet levels were 79 $\mu g/10^{10}$ platelets before taking Vitamin C and remained at this level at the end of the week. Platelet aggregation was nevertheless suppressed in

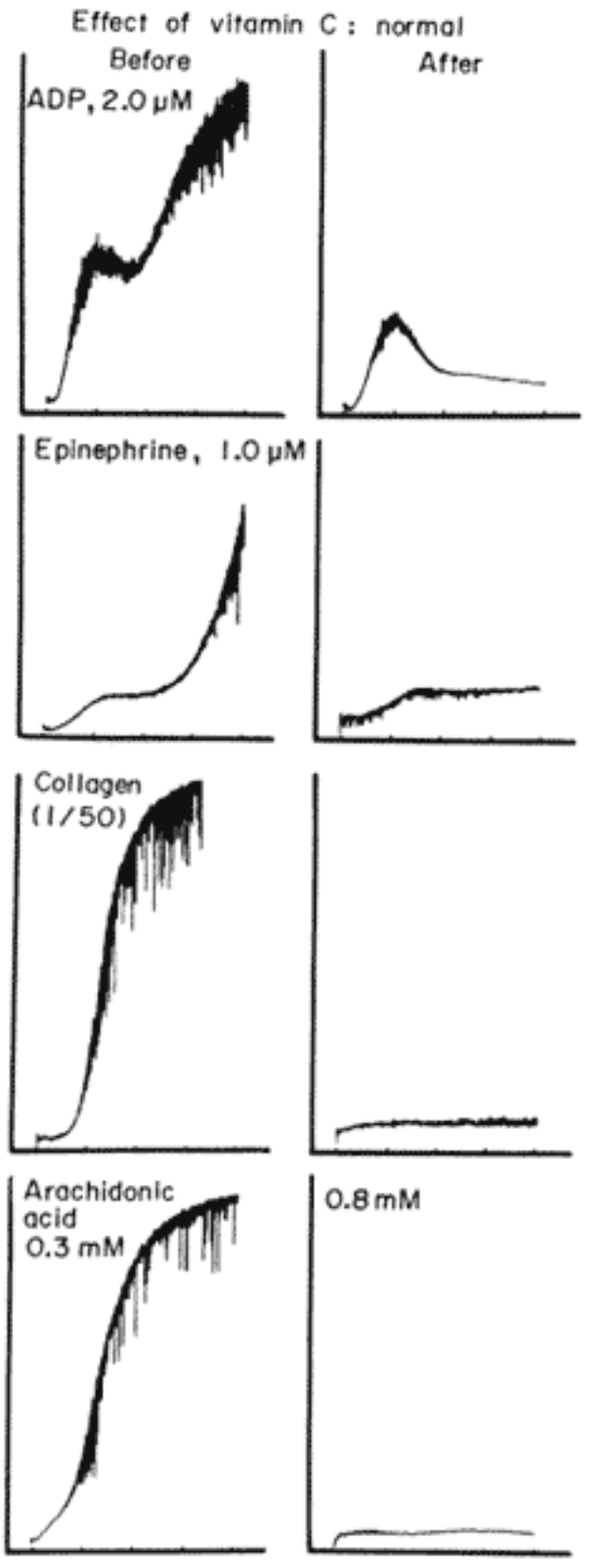

FIG. 4

Effect of oral Vitamin C for one week in a normal male on platelet aggregation.

this case with ADP and collagen, but increased with arachidonic acid. The other two normal subjects showed increased platelet levels of Vitamin C and suppression of aggregation with arachidonic acid as well as with the other aggregating agents.

A similar experiment was also performed on an insulin dependent diabetic. In this case, no change in aggregation was seen after Vitamin C (Figure 5).

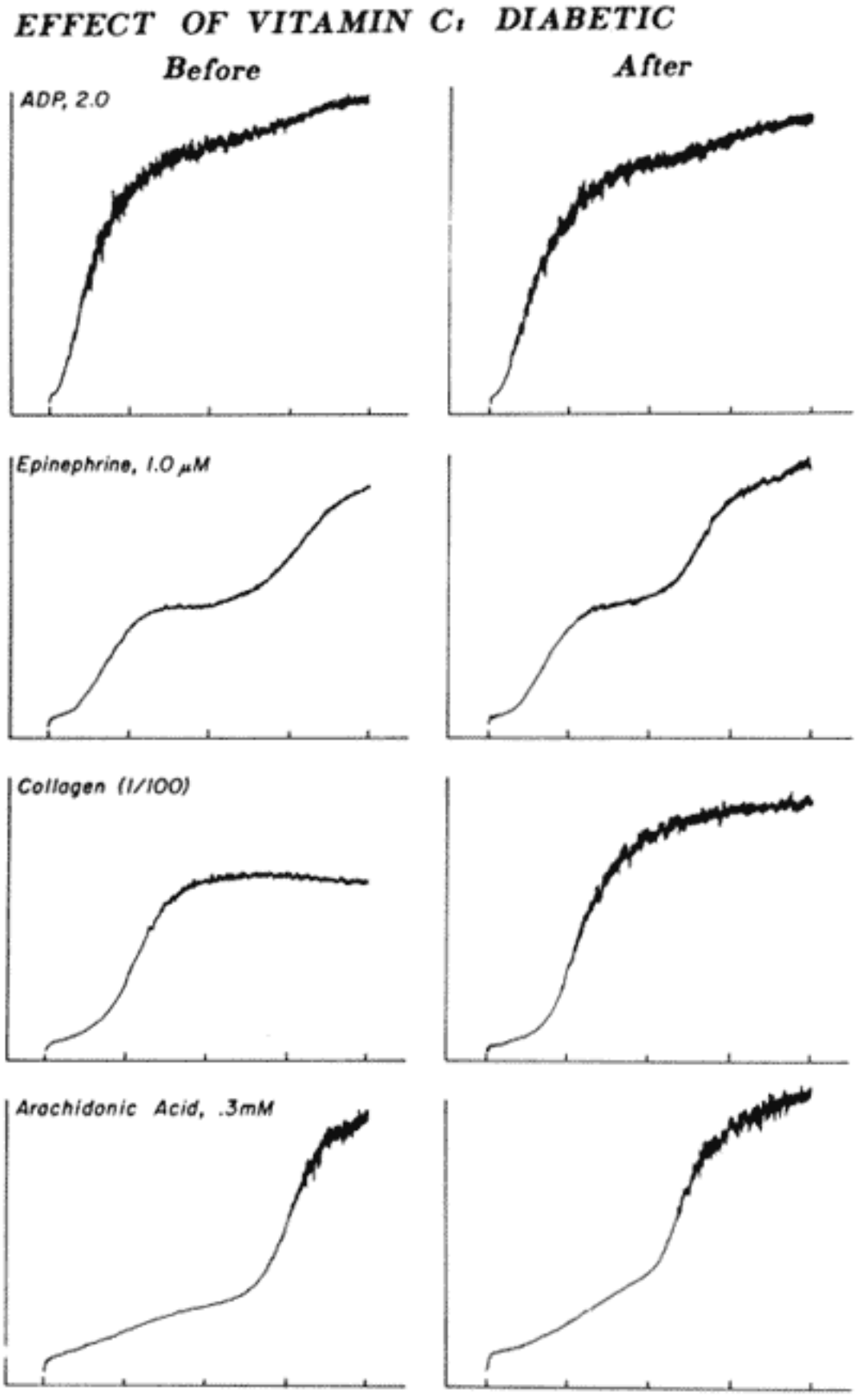

FIG. 5

Effect of oral Vitamin C for one week in a diabetic male on platelet aggregation.

TABLE 1

Effect of Oral Vitamin C on Platelet Aggregation

Aggregating Agent	N	Pre	Post	Paired t Test
ADP	6	51.8±8	17.5±8	$p < .025$
Epinephrine	5	53±11	24.6±17	$p < .1$
Collagen	5	51±17	5.4±2	$p < .05$
Arachidonic Acid	4	46±15	18±15	NS

DISCUSSION

Non-smoking insulin-requiring diabetics have lower platelet Vitamin C levels than normal non-smokers. Since plasma Vitamin C levels are normal in diabetics (23), it would appear that there may be a decreased transport of Vitamin C into the diabetic platelets, or an increased utilization. Mann (24) has suggested that the transport of Vitamin C may be facilitated by insulin and competitively inhibited by sugars. Insulin administration reduces the amount of plasma Vitamin C and is associated with a rise in the WBC/platelet concentration of Vitamin C (25). It has also been reported that sugars competitively inhibit the transport of dehydroascorbic acid into red blood cells (26).

There are several possible mechanisms through which ascorbic acid might inhibit platelet aggregation. Platelet Vitamin C might affect platelet prostaglandin production, since it forms an oxidation reduction system in the body. Certain anti-oxidants, e.g. α-tocopherol and propylgallate, have been shown to have inhibitory effects on the synthesis of prostaglandins (27) and platelet aggregation (28). Vitamin C affects prostaglandin production in two other tissues, guinea pig tracheal smooth muscle and guinea pig uterus (29,30). Vitamin C has also been shown to be inhibitory to cyclo-oxygenase activity (31).

Another possibility is that Vitamin C might raise cyclic-AMP levels. It

has been shown that Vitamin C can inhibit phosphodiesterase (32), thus preventing degradation of cAMP.

Clinically, Vitamin C has been shown in some cases to reduce the incidence of thrombosis and vascular disease. In one clinical study, the incidence of deep vein thrombosis in those at risk, as determined by I^{125} fibrinogen, was reduced by one half in subjects receiving Vitamin C (33). Knox (34) found strong negative correlation between ascorbate consumption and ischemic heart disease ($r = -.49$) and cerebrovascular disease ($r = -.68$).

It appears that the inhibition of platelet aggregation by Vitamin C may relate to these clinical findings. More work is needed before definitive statements can be made regarding the exact role of Vitamin C in platelet aggregation.

ACKNOWLEDGEMENTS

This work was supported by the Medical Research Service of the Veterans Administration, the American Diabetes Association, and the South Carolina State Appropriation for Biomedical Research. The excellent technical assistance of Susan Hosterman, Karen Bolton, and Sheryl Shaw is gratefully acknowledged.

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